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INCREASED HYDROGEN PEROXIDE LEVELS IN GLUCOSE 6-PHOSPHATE DEHYDROGENASE DEFICIENT ERYTHROCYTES EXPOSED TO ACETYLPHENYLHYDRAZINE

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Abstract—Acetylphenylhydrazine (APH), a classic hemolytic drug that produces hydrogen peroxide via a coupled oxidation with oxyhemoglobin, was incubated with normal and glucose 6-phosphate dehydrogenase (G6-PD)-deficient erythrocytes. Higher concentrations of H_2O_2 were attained in the G6-PD-deficient cells. The intracellular H_2O_2 levels were monitored by studying the formation of the enzyme–substrate complexes of catalase:

$$\text{Catalase} + H_2O_2 \rightleftharpoons \text{Complex I} \rightleftharpoons \text{Complex II}.$$

Two separate anticatalase effects of APH distinguished the two complexes; in both instances, the change in catalase activity was the dependent experimental variable that served as a convenient and specific amplifier of intracellular H_2O_2. The formation of Complex II was indicated by an inhibition of catalase, reversible by ethanol. The steady state concentration of Complex I was evaluated by measuring its rate of reaction with 3-amino-1,2,4-triazole, a reaction leading to irreversible inhibition. Greater anticatalase action by APH alone or by APH plus aminotriazole indicated higher concentrations of H_2O_2 in the G6-PD-deficient cells. These results support the theory that drug-induced hemolysis in G6-PD-deficient individuals involves oxidant damage by H_2O_2.

WHEN INDIVIDUALS with a genetically transmitted disorder known as "primaquine-sensitivity," i.e. a deficiency in erythrocyte glucose 6-phosphate dehydrogenase (G 6-PD) (D-glucose 6-phosphate: NADP oxidoreductase, EC 1.1.1.48), are treated with any of a wide variety of drugs (including aspirin, phenacetin, menadione, sulfonamides, nitrofurans, and the 8-aminoquinoline antimalarials like primaquine), their erythrocytes hemolyze.[1] The resulting anemia is associated with extensive oxidation of hemoglobin and is invariably preceded by a loss of reduced glutathione (GSH) from the erythrocytes. In normal erythrocytes, the first (G 6-PD) and second enzymatic reactions of the pentose phosphate pathway generate NADPH which can be utilized by glutathione reductase (NADPH: GSSG oxidoreductase, EC 1.6.4.2). The decline in GSH in G 6-PD-deficient cells can be attributed to the decreased production of NADPH with consequent impairment of the capacity to reduce oxidized glutathione. It has been suggested[2, 3] that the loss of GSH, by causing a decrease in the activity of glutathione peroxidase (GSH: H_2O_2 oxidoreductase, EC 1.11.1.9), predisposes the cells to oxidative damage by H_2O_2 produced by the drugs.

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A major prediction of this theory is that when G 6-PD-deficient and normal erythrocytes are exposed to H_2O_2-generating hemolytic drugs, higher intracellular concentrations of H_2O_2 should be attained in G 6-PD-deficient cells. We tested this hypothesis with acetylphenylhydrazine (APH), a classic hemolytic drug that generates H_2O_2 in erythrocytes[3] via a coupled oxidation with oxyhemoglobin and probably by autoxidation as well. We monitored the intracellular concentrations of H_2O_2 by studying the formation of the enzyme–substrate complexes of catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6). Higher concentrations of H_2O_2 were observed in the G 6-PD-deficient cells, as predicted by the theory.

METHODS

These experiments were performed with blood specimens from 16 normal subjects and 7 subjects deficient in G6-PD. Blood samples (10 ml) were drawn into heparinized vacutainers (Becton-Dickinson, N.J.) and centrifuged at 700 *g* for 10 min. Plasma and buffy coats were removed; the erythrocytes were washed three times with 3–4 vol. isotonic saline, and finally suspended at hematocrits of 50 per cent in saline–phosphate buffer,[4] pH 7·4, containing 22 mM glucose. Aliquots of 0·5 ml were then mixed with an equal volume of buffer (control) or buffer containing APH (acetylphenylhydrazine, i.e. acetophenylhydrazide, Eastman Organic Chemicals, New York). Final hematocrit was 25 per cent; glucose 11 mM; APH 16·7 mM. 3-Amino-1,2,4-triazole (AT, Mann Research Laboratories, New York) was added, where indicated, to a final concentration of 50 mM. After incubation at 37° for 2 hr, samples of the reaction mixture were diluted 20-fold in isotonic buffer; aliquots of these diluted samples were admixed either: (1) with a half volume of 0·45 M ethanol in isotonic buffer and held at room temperature for 20 min to reverse Complex II (see below); or (2) with a half volume of isotonic buffer and held at 0° to preserve Complex II. These samples were then lysed in 50 vol. distilled water at 0° and assayed in duplicate for catalase activity with a permanganate titration method.[5]

In a manner similar to that described in prior studies,[3, 5] intracellular H_2O_2 levels were monitored during incubation with APH by studying the formation of the enzyme–substrate complexes of catalase[6, 7] (Fig. 1). Complex I is the primary, reversible com-

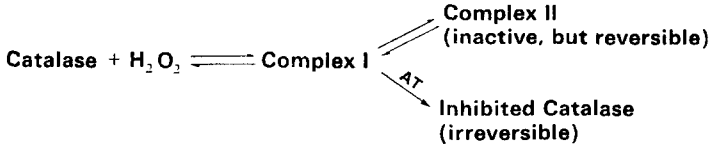

FIG. 1. The reactions of H_2O_2 with catalase. The abbreviation AT = 3-amino-1,2,4-triazole.

plex of catalase with H_2O_2. When it reacts with a second molecule of H_2O_2, as in the final assay system, oxygen is evolved, and this activity is defined as catalatic activity. Complex II, which is in equilibrium with Complex I, is characterized by an inability to react further with H_2O_2, i.e. it is catalatically inactive. This latter characteristic, the loss of enzymatic activity, provided a convenient endpoint in our experiments. Complex II can be decomposed by ethanol, however, and this leads to recovery of the catalatic activity. In these experiments, Complex II was defined operationally as that amount of catalatic activity which could be activated by treatment with ethanol. The

accumulation of Complex II reflected the intracellular concentration of free H_2O_2 in equilibrium with the catalase–H_2O_2 complexes.

In a second series of experiments, the steady state levels of Complex I in the incubation mixtures were separately measured by adding the catalase inhibitor aminotriazole (AT) at zero time; AT inhibits catalase[8] by reacting with Complex I (see Fig. 1). In these experiments, the inhibited catalase could not be reversed by ethanol, and this point verified the mechanism of inhibition as being "trapping" of Complex I rather than formation of Complex II. The rate of inhibition of catalase by AT is directly proportional to the rate of addition of H_2O_2 to the system.[3, 9] The rate of inhibition reflects the steady state concentration of Complex I in dynamic equilibrium with free intracellular H_2O_2.

RESULTS

The loss in catalase activity due to APH treatment was markedly greater in the G 6-PD-deficient cells than in the normal cells (Fig. 2A). G 6-PD-deficient cells exhibited losses averaging 54 per cent and normal cells exhibited losses averaging 5 per cent ($P < 0{\cdot}001$). The anticatalase effect was due to two phenomena: 1) the accumulation of the inactive enzyme–substrate complex of catalase (Complex II); and 2) a nonspecific loss in catalase activity.

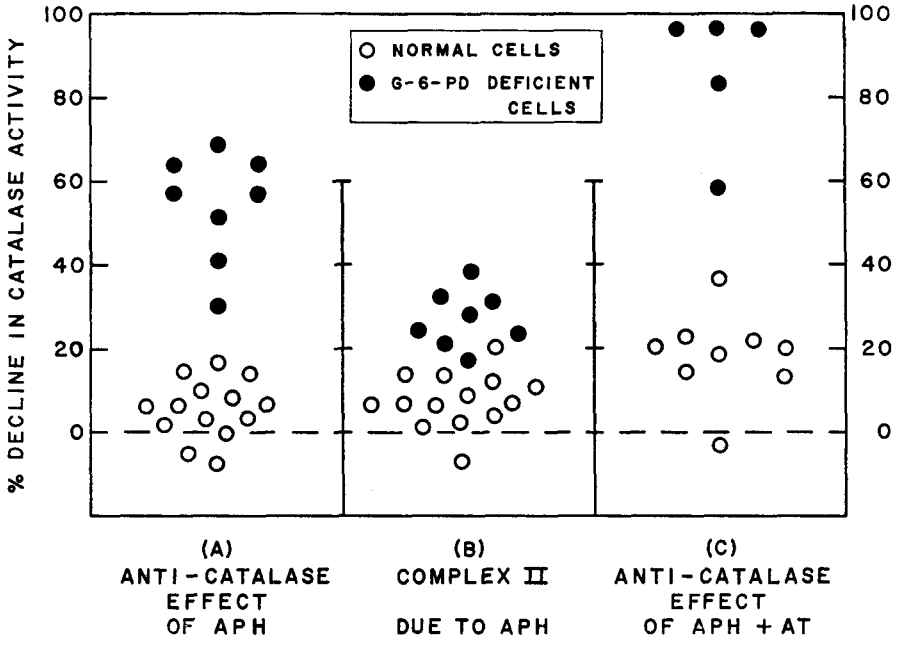

FIG. 2. The anticatalase effects of APH on intracellular erythrocyte catalase: (A) the total loss in catalase activity due to incubation with APH; (B) the loss attributed to the formation of the inactive Complex II; (C) irreversible loss due to reaction of the catalase inhibitor AT with Complex I.

Complex II levels were determined by measuring the increase in catalase activity after dissociation of the inactive Complex II to free, active catalase by treatment with ethanol. The zero time Complex II levels averaged 7 per cent (S.D. $\pm$ 4·8) for normals and 17·1 per cent (S.D. $\pm$ 7·2) for G 6-PD-deficient cells ($P < 0{\cdot}001$). The Complex II

values presented in Fig. 2B are the amounts generated by APH expressed as a percentage of total control activity, that is, experimental values minus control values (incubated without APH) divided by the total catalase activity (ethanol-treated) of the control. Average Complex II formation due to APH treatment was 7 per cent in the normal red cells and 27 per cent in the G 6-PD-deficient cells ($P < 0{\cdot}001$). The higher Complex II levels in the enzyme-deficient cells reflected the higher intracellular levels of free H_2O_2.

The small anticatalase effects noted in the normal cells (Fig. 2A) were completely attributable to Complex II accumulation. However, in the enzyme-deficient cells, a significant fraction of the lost catalase activity could not be recovered by treatment with ethanol. This nonspecific loss averaged 21 per cent, whereas normals exhibited an average gain of 2 per cent. Once again the G 6-PD-deficient cells were significantly more sensitive to this form of catalase loss ($P < 0{\cdot}001$).

In another series of experiments, samples from 8 normal subjects and 5 G 6-PD-deficient subjects were incubated with APH plus the catalase inhibitor AT. Since AT inhibits catalase by reacting with the primary enzyme–substrate complex (Complex I), this system provides a measure of the amount of Complex I present in the cells. In Fig. 2(C), the inhibition of catalase averaged 18 per cent for normals and 86 per cent for G 6-PD-deficient cells ($P < 0{\cdot}001$). These experiments provided independent evidence for increased steady state levels of H_2O_2 in G 6-PD-deficient cells during incubation with APH.

In a series of model experiments, we sensitized erythrocytes from 3 normal subjects by eliminating from the incubation mixture the glucose required for pentose shunt activity. These cells reacted similarly to the glucose-supplemented G 6-PD-deficient cells and exhibited anticatalase effects of 40, 53 and 70 per cent and AT inhibitions of 81, 98 and 98 per cent.

In a second series of model experiments, we sensitized erythrocytes from 3 normal subjects by treating the cells with *N*-ethylmaleimide (as described elsewhere[3]) to remove the intracellular GSH.[10] These cells exhibited anticatalase effects of 27, 35 and 43 per cent and AT inhibitions of 97, 100 and 100 per cent.

DISCUSSION

Our experiments with APH were modeled after those of Beutler *et al.*,[11] who studied the disappearance of GSH from G 6-PD-deficient cells. We confirmed the loss in GSH from the enzyme-deficient cells in several experiments. In G 6-PD-deficient cells, the diminished pentose shunt activity limits the amount of NADPH available for regenerating GSH from its oxidized form. Since the oxidation of GSH, catalyzed by GSH peroxidase, is the major reaction for detoxification of low concentrations of H_2O_2 within erythrocytes,[2] it follows that as the GSH level declines the steady state concentration of H_2O_2 should increase. This prediction was borne out in our experiments with APH. Loss of GSH occurs as a prelude to erythrocyte destruction when sensitive (i.e. enzyme-deficient) subjects are exposed to certain drugs.[1] A build-up in intracellular H_2O_2 *in vivo* should lead to increased oxidative damage, and this apparently culminates in erythrocyte destruction.

Recently, Nicholls[12] studied the activity of catalase in intact, glucose-deprived *horse* erythrocytes that had been stored in the glucose-deprived condition at 4° for several

days. The levels of GSH and the activity of GSH peroxidase in these cells were unknown. Nicholls concluded[12] that at all concentrations of H_2O_2, catalase would react with H_2O_2 more quickly than GSH peroxidase by a factor of 10^4. Presumably then, elimination of GSH peroxidase activity could not produce a detectable change in the rate of reaction of catalase with H_2O_2. However, in our experiments with APH, we observed increased intracellular concentrations of the enzyme–substrate complexes of catalase in the G 6-PD-deficient cells. Our results must be ascribed to the decline in GSH peroxidase activity, since similar effects were obtained with normal erythrocytes when they were deprived of GSH by (1) treatment with N-ethylmaleimide or (2) by withholding the glucose required for pentose shunt activity. Furthermore, these results cannot be ascribed to some as yet unclarified effect of APH in G 6-PD-deficient cells, since results similar to ours were obtained by Bracci *et al.*[13] when gaseous H_2O_2, added by a diffusion method, was used instead of APH. These results are consistent with the viewpoint that in human erythrocytes GSH peroxidase activity sustained by the NADPH-dependent glutathione reductase is a major route for the detoxification of low concentrations of H_2O_2.

In our experiments, the anticatalase action of APH on G 6-PD-deficient cells was shown to be due to the formation of Complex II and to a nonspecific loss in catalase. The same mechanisms were no doubt involved in (1) the 20–30 per cent losses in catalase activity reported by Ezra *et al.*[14] for G 6-PD-deficient cells exposed to APH or primaquine, and (2) the anticatalase effect on G 6-PD-deficient cells of plasma drawn after ingestion of primaquine reported by Tarlov *et al.*[15] Formation of H_2O_2 in intact erythrocytes incubated with primaquine or other hemolytic agents has been reported.[5]

A measure of the greater sensitivity of G6-PD-deficient cells to the effects of H_2O_2 may be derived from the nonspecific loss (i.e. irreversible by standard 20-min treatment with ethanol) of catalase. We suggest the existence of a mechanism which limits the accumulation of Complex II—perhaps the conversion of Complex II into the inactive Complex III, a reaction which is known[7] to be catalyzed specifically by H_2O_2. Alternatively, the irreversible loss of catalase activity may be due to oxidation of the enzyme–protein by the H_2O_2 present in higher concentration in the G 6-PD-deficient cells. In either event, the phenomenon did not occur to any significant extent in the normal cells in which the entire anticatalase effect of APH was accounted for by Complex II formation.

Tarlov *et al.*[15] observed an unexplained anticatalase effect during primaquine-induced hemolysis in G 6-PD-deficient subjects. Normal subjects were insensitive to both hemolysis and catalase decline. An explanation for this observation, consistent with the results we report here, is that the H_2O_2 produced by primaquine or its metabolites resulted in the formation of the inactive Complex II *in vivo*.

Tarlov and Kellermeyer[16] reported decreased average erythrocyte catalase levels in unstressed G 6-PD-deficient subjects. This report conflicted with an earlier survey by Beutler *et al.*[17] Ezra *et al.*[14] reinvestigated this point but could not detect any difference between normal and G 6-PD-deficient cells, and even Tarlov and Kellermeyer could not duplicate their finding at a later time.[16] However, we observed significantly higher average levels of Complex II in G6-PD-deficient cells at zero time. Since accumulation of Complex II is in fact an anticatalase action, it is conceivable that at times the effect may be strong enough to be detectable as an overall diminution in catalase levels. Observation of a strong decline may depend upon ancillary factors

such as diet, the avoidance of ethanol as an antiseptic for venipuncture (since it decomposes Complex II), and other factors in the details of preparing samples for assay. The zero time Complex II levels are most probably due to endogenous peroxide-generating agents (such as ascorbate, foodstuffs).

Although the greater Complex II levels we observed at zero time were small, they were significant and consistent with the small but significant diminution in GSH levels reported for G 6-PD-deficient cells by Beutler *et al.*[17] The validity of this latter observation was borne out by the development of the GSH-stability test (APH-stress), which subsequently led to the elucidation of G 6-PD deficiency as the primary defect in primaquine-sensitive cells.

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